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Metabolic engineering of oilseed crops to produce high levels of novel acetyl glyceride oils with reduced viscosity, freezing point and calorific value

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Summary

Seed oils have proved recalcitrant to modification for the production of industrially useful lipids. Here, we demonstrate the successful metabolic engineering and subsequent field production of an oilseed crop with the highest accumulation of unusual oil achieved so far in transgenic plants. Previously, expression of the *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) gene in wild-type Arabidopsis seeds resulted in the accumulation of 45 mol% of unusual 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) in the seed oil (Durrett *et al.*, 2010 PNAS 107:9464). Expression of *Ea*DAcT in *dgat1* mutants compromised in their ability to synthesize regular triacylglycerols increased acetyl-TAGs to 65 mol%. Camelina and soybean transformed with the *Ea*DAcT gene accumulate acetyl-triacylglycerols (acetyl-TAGs) at up to 70 mol% of seed oil. A similar strategy of coexpression of *Ea*DAcT together with RNAi suppression of *DGAT1* increased acetyl-TAG levels to up to 85 mol% in field-grown transgenic Camelina. Additionally, total moles of triacylglycerol (TAG) per seed increased 20%. Analysis of the acetyl-TAG fraction revealed a twofold reduction in very long chain fatty acids (VLCFA), consistent with their displacement from the *sn*-3 position by acetate. Seed germination remained high, and seedlings were able to metabolize the stored acetyl-TAGs as rapidly as regular triacylglycerols. Viscosity, freezing point and caloric content of the Camelina acetyl-TAG oils were reduced, enabling use of this oil in several nonfood and food applications.

Introduction

3-Acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) are unusual triacylglycerols with an *sn*-3 acetyl group instead of a fatty acyl group. The presence of the *sn*-3 acetyl group confers useful physical, chemical and nutritional properties to these molecules. For example, they possess reduced kinematic viscosity (Durrett *et al.*, 2010) and lower melting points (Marshall *et al.*, 2014) compared to conventional triacylglycerols (here referred to as lcTAGs). Acetylglyceride mixtures produced synthetically are used in a variety of food and industrial applications, including as emulsifiers, food coatings and plasticizers (Gaupp and Adams, 2004).

Seeds of *Euonymus alatus* (Burning Bush) accumulate acetyl-TAGs as the major component of their storage oil. Previously, we identified an acetyltransferase, *Ea*DAcT, from *E. alatus* that synthesizes acetyl-TAGs from DAG and acetyl-CoA *in vitro* and results in up to 45 mol% acetyl-TAG when expressed in wild-type Arabidopsis seeds (Durrett *et al.*, 2010). *Ea*DAcT is a member of the membrane-bound O-acyltransferase (MBOAT) family, a group of acyl-CoA-dependent enzymes that acylates hydroxyl groups of lipids and proteins (Hofmann, 2000). Important members of this family also include the type 1 diacylglycerol acyltransferases (DGAT1) which synthesize lcTAGs in animals and plants (Liu *et al.*, 2012). Although *Ea*DAcT and DGAT1 share a common substrate, the two enzymes are only distantly related among the MBOAT family. Instead, *Ea*DAcT clusters more closely with a wax synthase and a sterol acyltransferase (Figure S1).

Here, we provide evidence that down-regulation of the DGAT1 pathway for synthesis of lcTAGs leads to the enhanced accumulation of acetyl-TAGs in Arabidopsis and Camelina. The successful application of this strategy to the oilseed crop *Camelina sativa* resulted in up to 85 mol% acetyl-TAGs in the oil, representing the highest levels of unusual lipids achieved in transgenic plants. The accumulation of these structurally different storage lipids by field-grown plants had minor or no impact on seed size, oil content, germination or oil mobilization by seedlings. In a concurrent study (Liu *et al.*, 2015) we obtained similar results from transformations of a high-oleic Camelina line. Analysis of acetyl-TAGs from field-grown plants demonstrated significant and useful differences in the physical properties of this unusual oil.

Results and discussion

Accumulation of acetyl-TAGs is enhanced in the Arabidopsis *dgat1* background

Expression of *Ea*DAcT in wild-type Arabidopsis seeds results in the accumulation of up to 45 mol% acetyl-TAGs in the seed oil (Durrett *et al.*, 2010). We reasoned that *Ea*DAcT likely competes with endogenous TAG biosynthetic enzymes for DAG substrate, limiting the accumulation of acetyl-TAG. Diacylglycerol acyltransferase (DGAT1) is responsible for the majority of conversion of

DAG to lcTAG in Arabidopsis (Zhang *et al.*, 2009). To test whether the elimination of DGAT1 would lead to higher acetyl-TAG levels, we expressed the *Ea*DAcT acetyltransferase in an Arabidopsis *dgat1* mutant. In the mutant background, acetyl-TAG levels increased to 60–65 mol% of total TAGs in the highest accumulating lines, compared to 45 mol% in the Col-0 wild-type background (Figure 1a). These high levels of acetyl-TAGs in the transgenic seed oil were confirmed by quantifying the transgenic oil composition with electrospray ionization–mass spectrometry (ESI-MS; Figure S2).

The seed oil of Arabidopsis *dgat1* mutants possesses an altered fatty acid profile, including a reduction in very long-chain fatty acids (VLCFA; Katavic *et al.*, 1995; Routaboul *et al.*, 1999). As the carbon atoms for the elongation reaction that synthesizes VLCFA are ultimately derived from cytosolic acetyl-CoA, it is possible that the *dgat1* mutants possess higher levels of this substrate for the activity of *Ea*DAcT. However, such potential increases in cytosolic acetyl-CoA would not fully explain the increase in acetyl-TAGs in the *dgat1* background: compared to *Ea*DAcT expressing plants in a wild-type background, VLCFA are only reduced by 80 nmoles/mg DW, whereas acetyl-TAG levels increased by approximately 250 nmoles/mg DW. The increased levels of acetyl-TAGs when DGAT1 function is eliminated implies that even though *Ea*DAcT and DGAT1 have only 32% sequence identity and are predicted to possess different membrane topologies, both enzymes likely access an endogenous pool of DAG that is available for TAG synthesis.

Production of very high levels of acetyl-TAGs in Camelina

To produce larger quantities of acetyl-TAGs to evaluate physical, chemical and functional properties, we adopted a similar strategy of combining *Ea*DAcT expression with suppression of endogenous lcTAG synthesis in the oilseed crop *C. sativa*. A member of the Brassicaceae family, Camelina, is particularly suitable for the production of industrial and biofuel feedstocks as it is currently not widely used as a food crop and requires less agricultural inputs than other oilseed crops (Putnam *et al.*, 1991; Zubr, 1997). Camelina mutants in enzymes responsible for lcTAG assembly are not available; additionally, as Camelina possesses an allohexaploid genome, up to three homoeologues of each enzyme are expressed in seeds (Hutcheon *et al.*, 2010). Therefore, we designed an RNAi hairpin loop driven by a strong seed-specific oleosin promoter to simultaneously suppress expression of all three Camelina *DGAT1* homoeologues. Another strong seed-specific promoter, that of the soybean glycinin gene, was used to express the *Ea*DAcT coding sequence, either by itself or with RNAi to target the Camelina *DGAT1* genes (Figure S3). Additional constructs also included RNAi for the phospholipid:diacylglycerol acyltransferase (*PDAT1*) gene, another acyltransferase capable of synthesizing lcTAG (Zhang *et al.*, 2009), but the results were not statistically different from those with RNAi for *DGAT1* alone (data not shown). At this point, it is unclear why higher acTAG levels were not achieved with RNAi of both *DGAT1* and *PDAT1*. One potential explanation is because both *DGAT1* and *PDAT1* are required for embryo development in Arabidopsis (Zhang *et al.*, 2009), the strong and simultaneous down-regulation of both genes in Camelina might be detrimental to overall seed development. Alternatively, sufficient suppression of *PDAT1* transcript levels may not have been achieved. In the future, careful titration of *DGAT1* and *PDAT1* expression might represent a more effective strategy to further decrease lcTAG levels.

Expression of *Ea*DAcT alone resulted in acetyl-TAG levels of 47–64 mol% in independent Camelina lines (Figure 1b). Furthermore, the strategy of inhibiting endogenous lcTAG synthesis was successful in increasing the levels of the target oil: coexpression of

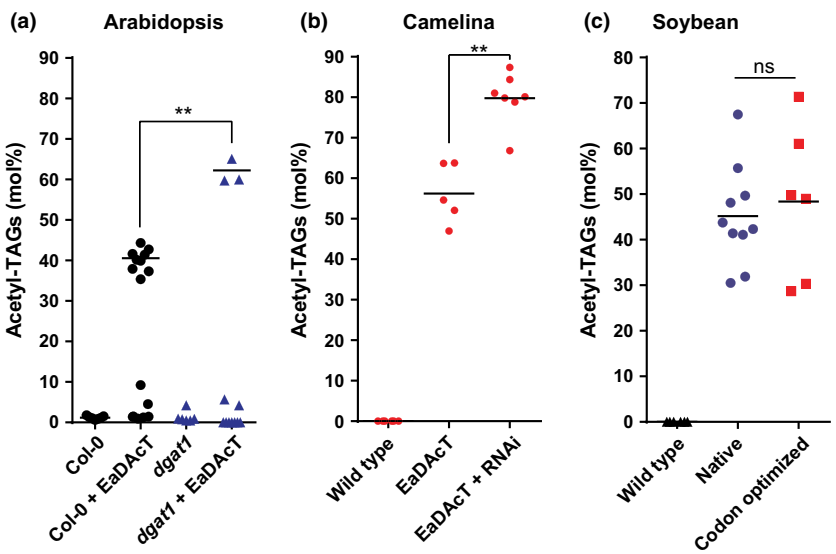

Figure 1 Simultaneous expression of *Ea*DAcT and down-regulation of endogenous lcTAG synthesis increases acetyl-TAG accumulation in seeds. (a) Scatter plot of the distribution of acetyl-TAG levels of homozygous T_3 seed from individual transgenic Arabidopsis Col-0 wild-type or *dgat1* lines expressing *Ea*DAcT. (b) Scatter plot of the distribution of acetyl-TAG composition of T_2 seed from independent transgenic Camelina lines transformed with *Ea*DAcT alone and with RNAi constructs targeting Camelina *DGAT1* homoeologues. Null segregants were removed from T_2 seeds based on the absence of dsRED visual transformation marker. (c) Scatter plot of the distribution of the acetyl-TAG composition from T_1 soybean seeds expressing either the native *Ea*DAcT gene or a codon-optimized version. Horizontal lines represent the mean value for each sample group. Asterisks indicate significant difference ($P < 0.01$; Mann–Whitney *U*-test). ns, not significant ($P = 0.706$, Mann–Whitney *U*-test).

*Ea*DAcT together with RNAi of *DGAT1* resulted in acetyl-TAG levels of approximately 85 mol% in the best lines (Figure 1b), a level higher than those obtained in previous efforts to engineer novel oil compositions in oilseeds. These very high levels of acetyl-TAGs in the transgenic oil were additionally confirmed using ESI-MS (Figure 2a). When subjected to ESI-MS^2, molecular ion peaks corresponding to acetyl-TAGs underwent a loss of ammonium acetate (Figure 2b), confirming the identity of these molecules.

In species of the plant family Brassicaceae, the VLCFA are predominantly found at *sn*-1 and *sn*-3 positions of TAG (Takagi and Ando, 1991; Taylor *et al.*, 1995). Compared to the wild-type oil content, and to lcTAGs of the transgenic plants, the fatty acid composition of the acetyl-TAGs contained an approximately twofold reduction of VLCFA and was enriched in the polyunsaturated fatty acids 18:2 and 18:3 (Figure 2c). The reductions of VLCFA from acetyl-TAG are consistent with their expected enrichments at *sn*-3 (for 20:0) and *sn*-1 + *sn*-3 positions (for 20:1 and 22:1) in lcTAGs (Takagi and Ando, 1991; Taylor *et al.*, 1995).

Production of high levels of acetyl-TAG in field-grown Camelina

To determine whether Camelina is able to produce high levels of acetyl-TAG under field conditions and to obtain enough oil for property testing, seeds from independent transformation events that displayed the highest acetyl-TAG/lcTAG ratios were multiplied in the greenhouse and planted in the field. The levels of acetyl-TAG for the different transgenic lines grown in the field were similar to those observed in the seed from growth-chamber-grown plants. Seed from replicate plots of a line expressing only *Ea*DAcT had an average acetyl-TAG composition of 64.6 mol% (SE = 0.57%). For the three independent lines that included suppressed expression of *DGAT1*, the acetyl-TAG levels were higher at 86.2, 82.7 and 74.4 mol% (SE = 0.11, 1.27 and 2.73%, respectively; Figure 3a). These levels compare well to those of the original seed from which these lines were derived (Figure 1b), indicating that the trait is stable until at least the T_5 generation. This stability of the high acetyl-TAG phenotype over multiple generations is of obvious importance from a biotechnology perspective as it simplifies the production of seed for planting and allows for crossing into additional germplasm varieties without loss of phenotype.

The transgenic seeds accumulated more moles of TAG compared to wild-type seed (Figure 3a). We observed 15–21% increases in total moles of TAG per seed dry weight in the lines expressing *Ea*DAcT. Biosynthesis of one mole of lcTAG requires three moles of fatty acyl-CoA, whereas synthesis of acetyl-TAG requires only two moles, and one mole of acetyl-CoA. Therefore, stoichiometrically 50 mol% more TAG can be produced if fatty acid synthesis remains constant and all fatty acids destined for lcTAG are incorporated into acetyl-TAG. This increase in moles of TAG would occur only if the fluxes of cytosolic glycerol 3-phosphate and acetyl-CoA were not limiting in the transgenic seeds. However, for seeds producing 80 mol% acetyl-TAG, the observed increase of approximately 20% (Figure 3a) is lower than the 36.7 mol% expected if net fatty acid accumulation remained constant. This result suggests that engineering an increased supply of glycerol 3-phosphate or acetyl-CoA may provide a strategy for synthesizing even higher acetyl-TAG levels.

Accumulation of high levels of acetyl-TAGs had no major impact on key traits of Camelina seeds grown in the field, and there were no visible differences in plant height or morphology between the transgenic lines expressing *Ea*DAcT and the wild-type controls. The average seed weights of *Ea*DAcT, *Ea*DAcT combined with RNAi and control lines were 1.37, 1.39 and 1.36 mg, respectively (Figure 3b). When calculated on a mass basis, the oil content of transgenic seeds from field-grown Camelina expressing only *Ea*DAcT and producing 70 mol% acetyl-TAG was 37.4 $\pm$ 0.8% of DW, not statistically different to that of control lines at 38.9 $\pm$ 0.4% (Figure 3c). However, the oil content of seeds that produced over 80 mol% acetyl-TAG and that were transformed with *Ea*DAcT and RNAi of *DGAT1* was reduced to between 34.6 $\pm$ 0.4% and 36.0 $\pm$ 0.5% (a 7.5–11% reduction; Figure 3c). Arabidopsis *dgat1* mutant seeds also possess lower oil content compared to wild type (Katavic *et al.*, 1995; Routaboul *et al.*, 1999), a phenotype which is not restored by the expression of *Ea*DAcT (Figure S2b). A parallel situation is possible in Camelina as we observed significant reductions in oil content only in the Camelina lines expressing *Ea*DAcT together with *DGAT1* RNAi suppression (Figure 3c). Such reductions may be an acceptable trade off for a value-added functional property and may be minimized by breeding and biotechnology-based approaches. Indeed, it is interesting to speculate that the inability of EaDAcT to complement the reduction of DGAT activity is due to limiting amounts of glycerol3-phosphate or acetyl-CoA. As noted above, engineering higher levels of either precursor therefore also represents one strategy to address the lower oil content.

Germinating seedlings efficiently metabolize acetyl-TAGs

As the structure and physiochemical properties of acetyl-TAG are different from the lcTAG encountered by Camelina lipases during germination, we asked whether acetyl-TAGs could be readily metabolized as a source of energy and biosynthetic precursors. In transgenic seedlings, both acetyl-TAG and lcTAG were completely metabolized within 7 days, a time frame equivalent to lcTAG metabolism in wild-type seedlings (Figure 4). These results indicate that endogenous seed TAG lipases can metabolize both classes of TAGs, and therefore, acetyl-TAG-specific lipases are not required for access to all stored fatty acyl chains and successful seedling growth. Consistent with these results, germination rates of seeds from field-grown lines were typically not reduced by the presence of these engineered lipids. For example, wild-type seeds germinated at a rate of 90%, whereas seed expressing only *Ea*DAcT germinated at a rate of 94%; of the three lines with both *Ea*DAcT and RNAi, two germinated at a rate of 86%. The third line with both *Ea*DAcT and RNAi possessed a germination rate of only 50%. However, as the same gene combination did not affect the other two lines, this low rate is most likely caused by deleterious insertion effects of the transgenic construct.

Production of acetyl-TAGs in soybean

Arabidopsis and Camelina are very closely related members of the Brassicaceae. It was therefore of interest to determine whether *Ea*DAcT could produce acetyl-TAG when expressed in a noncruciferous oilseed. We transformed soybean with both native and soy codon-optimized versions of *Ea*DAcT under the control of a seed-specific promoter. Analysis of neutral lipids extracted from segregating T_1 seeds revealed that many independent lines accumulated high levels of acetyl-TAG (Figures 1c and S4a,b). For some lines, these levels were as high as 70 mol%. There was no difference in acetyl-TAG production between native and codon-optimized versions of *Ea*DAcT (Figure 1c; $P = 0.706$, Mann–Whitney *U*-test). Relative to the lcTAGs, the acetyl-TAGs were

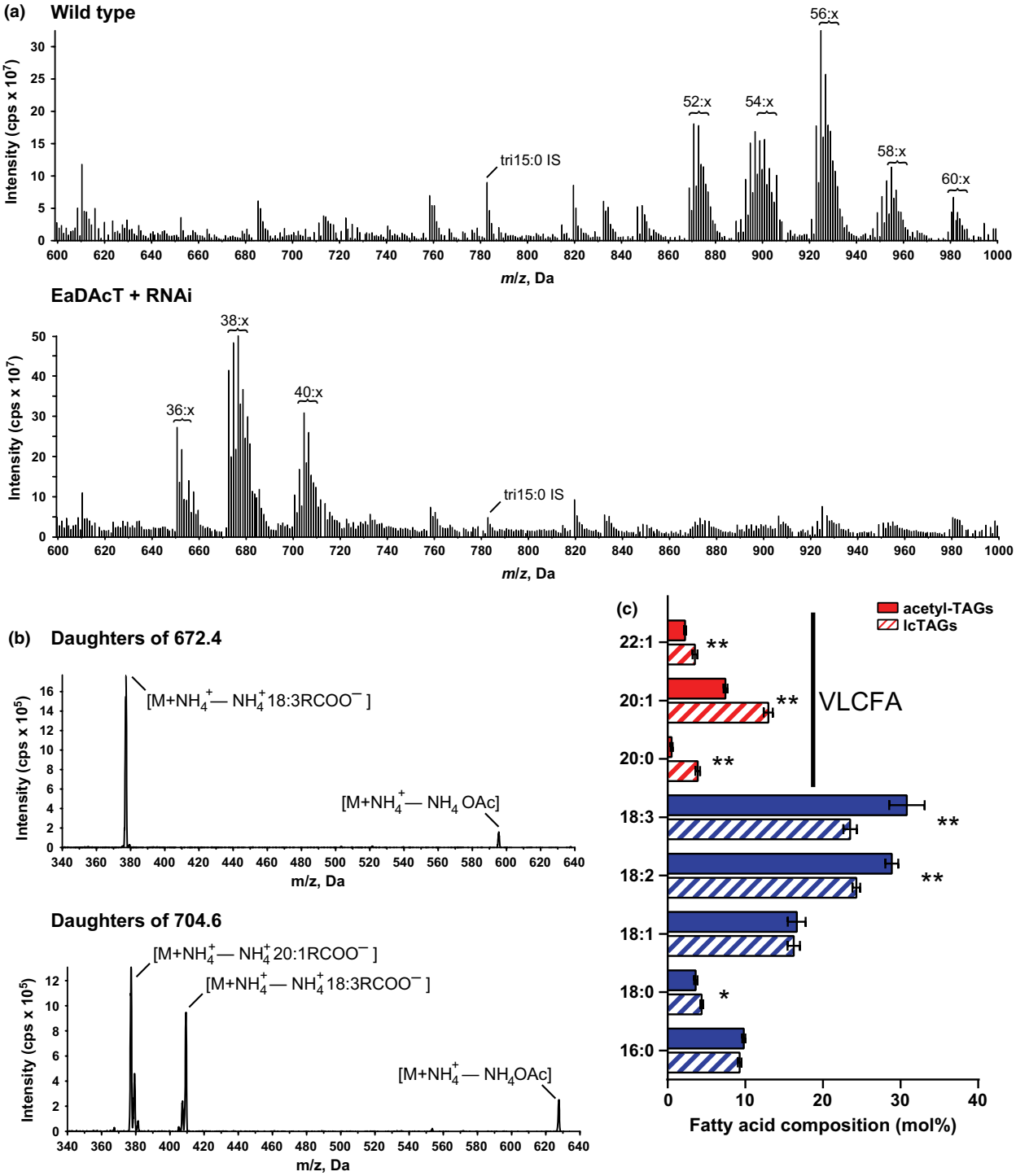

Figure 2 Camelina seeds expressing *Ea*DAcT accumulate acetyl-TAGs. (a) Positive ESI mass spectra of neutral lipid extracts from Camelina wild-type seed or transgenic T_2 seed expressing *Ea*DAcT. Signal peaks possess the *m/z* value of the $[M + NH_4]^+$ adduct. For clarity, only the number of acyl carbons and not the number of double bonds (x) in each series of TAG molecular species is indicated. Tripentadecanoin (tri15:0) was added as an internal standard during seed lipid extraction. (b) ESI-MS^2 daughter scans of acetyl-TAGs from Camelina seed expressing *Ea*DAcT. Shown are the fragment peaks from acTAGs with $[M + NH_4]^+$ adducts with masses of 672.4 and 704.6. (c) Mean fatty acid composition of acetyl-TAG and lcTAG fractions from the T_2 seed of five independent transgenic lines expressing *Ea*DAcT. Error bars represent SEM. Asterisks indicate statistical difference (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$).

enriched in 18:2 at the expense of the other fatty acids, particularly 16:0 and 18:0 (Figure S4c). Similar to the situation with Arabidopsis and Camelina, the soybean acetyl-TAG fatty acid composition reflects the loss of the *sn*-3 position for fatty acylation. This position accommodates half the saturated fatty acids in wild-type soybean lcTAGs (Takagi and Ando, 1991).

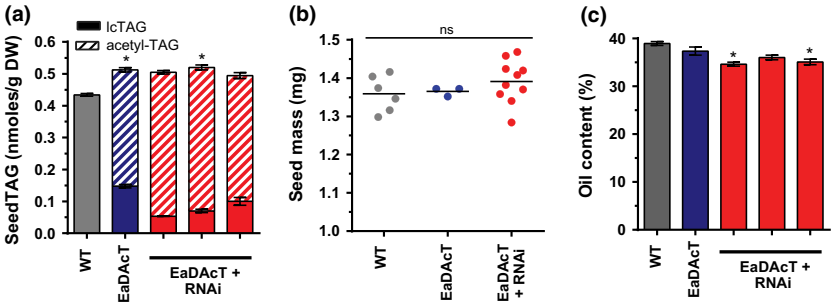

Figure 3 Accumulation of high levels of acetyl-TAGs has no major impact on key performance traits of Camelina grown in the field. Three independent transgenic lines combining *Ea*DAcT expression with RNAi of DGAT1 and one line with only *Ea*DAcT expression were grown in the field. (a) The mean TAG content of the field-grown seed is shown divided into acetyl- and lcTAG fractions. Error bars represent the SEM for the acetyl-TAG and lcTAG content of at least three replicate field plots. (b) The average mass of wild-type and transgenic Camelina seed was determined by weighing 50 seeds. Each data point represents seed from one 24 m^2 plot; the data from the three independent *Ea*DAcT + RNAi lines have been combined into one column. Horizontal lines represent the mean value for each sample group. (c) Mean oil content for wild-type and transgenic Camelina seed harvested from at least three different field plots was determined gravimetrically. Error bars represent the SEM. (Statistical analyses: (a, c) asterisks indicate statistically significant differences, $P < 0.05$; Kruskal–Wallis test with Dunn's multiple comparisons test (b); ns, not significant, $P = 0.448$, Kruskal–Wallis test).

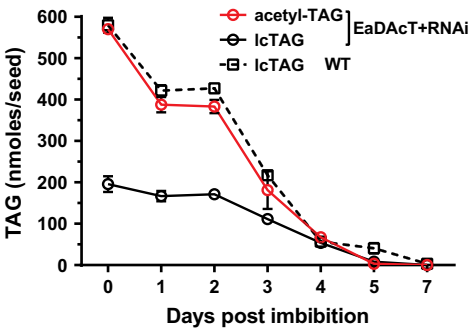

Figure 4 Acetyl- and lcTAG content of germinating wild-type Camelina seeds and transgenic seeds containing 80 mole% acetyl-TAGs. Error bars represent ± standard deviation of five replicates from one representative experiment.

Physical properties of transgenic acetyl-TAGs

To determine whether acetyl-TAG produced by expression of *Ea*DAcT in Camelina possesses reduced viscosity, we measured the kinematic viscosity (according to ASTM D455) of lcTAG purified from wild-type Camelina oil and of acetyl-TAG purified from *Ea*DAcT-transformed seed oil. The viscosity of lcTAG was 30.6 mm^2/s, in keeping with the literature values (Bernardo *et al.*, 2003; Paulsen *et al.*, 2011). In contrast, the kinematic viscosity of acetyl-TAG was 20.3 mm^2/s, a 34% reduction. This viscosity falls within the range specified for diesel #4 fuel (5–24 mm^2/s at 40 °C), used in the low- and medium-speed engines found in some locomotives or stationary power plants, and may allow the oil to perform as a 'drop-in' diesel fuel without transesterification. Vegetable oils also have a number of favourable properties as biodegradable lubricants (Cermak *et al.*, 2013) and the lower viscosity of acetyl-TAG may also expand these uses.

Poor cold temperature properties of traditional vegetable oils often limit their use in fuel, lubricant and other applications. To determine the thermotropic behaviour of acetyl-TAG compared to conventional vegetable oils, differential scanning calorimetry (DSC) was performed on Camelina acetyl-TAG and lcTAG and on synthetic *sn*-3-acetyl-1,2-diolein and triolein standards. Crystallization of TAGs, even of completely defined single molecular species, is a complex process involving several different polymorphic forms. Triolein exhibited a strong crystallization/freezing exotherm at −32.4 °C on cooling (corresponding to the α-polymorphic form) and a strong endotherm at 4.6 °C on heating, corresponding to melting of the stable β′-crystal form (Figure 5a,b). These transition temperatures are similar to those reported in the literature (Hagemann *et al.*, 1972). Substitution of oleoyl at the *sn*-3 position by acetyl greatly reduced the temperature during cooling for the strong crystallization/freezing exotherm by more than 20 °C, to −58.9 °C and slightly reduced the temperature for the strong endotherm on heating (to 3.6 °C). Additional transitional endothermic–exothermic polymorphic changes begin to occur for acetyldiolein at about −45 °C on melting, again, much lower than for similar events in triolein (Figure 5b). DSC analysis of vegetable oils with multiple saturated and unsaturated fatty acids would be expected to produce broader transitions, with reduced transition peak temperatures as unsaturation increases. Thus, for lcTAG from Camelina, which is more highly unsaturated compared to triolein, the transition temperatures for both the exotherm on cooling and the endotherm on heating are reduced substantially, by −18.1 °C and −27 °C, respectively (Figure 5c,d). An extremely broad but weak crystallization exotherm is also discernable over the −10 °C to −50 °C range; additional transitions are also present on melting. For acetyl-TAG from Camelina, the strong cooling exotherm corresponding to crystallization of the α-polymorphic form occurs at −68.8 °C, 18.3 °C lower than that for Camelina lcTAGs; the weaker cooling exotherm is more pronounced and also at a lower temperature (Figure 5c). There are multiple melting curve transitions, with the largest endotherm for acetyl-TAG about 16 °C lower than for lcTAG (Figure 5d). Whether these results presage improved applied low-temperature properties, such as pour points, will be important to determine.

The introduction of acetyl-TAG into edible oilseed crops such as soybean can also provide modified oils for human consumption. Synthetic acetylated mono- and diacylglycerols are recognized as safe and widely used for a number of applications in the food processing industry including as emulsifiers, food coatings and foam stabilizers (Gaupp and Adams, 2004; Lundsgaard *et al.*,

2009), and as plasticizers for packaging of food products (Coltro *et al.*, 2014). Additionally, the replacement of a long-chain fatty acid by acetic acid also means that acetyl-TAGs are reduced-calorie oils. The heats of combustion determined by bomb calorimetry were 9.62 ± 0.10 and 9.03 ± 0.05 kcal/g, respectively, for wild-type Camelina oil and the acetyl-TAG fraction from transgenic Camelina seeds. Thus, the absence of a third long acyl chain reduces the total calorific content of acetyl-TAGs by 6.3%. The actual calories available for deposition in adipocytes will be lower because acetate will not be metabolically converted to fat.

In conclusion, by combining the expression of *Ea*DAcT with the RNAi suppression of endogenous lcTAG synthesis, we have successfully engineered the synthesis of very high levels of acetyl-TAG in multiple plant species. We have also obtained similar results in a high-oleic line of Camelina (Liu *et al.*, 2015). The metabolic engineering of seeds to synthesize oils with novel fatty acids or wax esters at levels useful for industry has in most cases proved particularly challenging (Cahoon *et al.*, 2007). As *Ea*DAcT functions at the end of a biosynthetic pathway and utilizes a ubiquitous substrate (acetyl-CoA), the bottlenecks typically associated with the flux of unusual fatty acids from the site of their synthesis to storage as triacylglycerols are avoided. Indeed, the ability to synthesize 85 mol% acetyl-TAGs indicates plasticity in the supply of cytosolic acetyl-CoA.

Importantly, the synthesis of high levels of acetyl-TAG leads to an overall increase in the moles of oil produced without affecting seed size and viability, demonstrating that transgenic seeds can accommodate a substantial change in the size of the storage lipid molecule and its physical properties without negative impacts. The resulting oil has lower viscosity, freezing point and caloric content than current commodity oilseeds. Field production of this novel oil provides an abundant source of acetyl-TAG whose physiochemical properties can now be further characterized in much greater detail.

Experimental procedures

Arabidopsis transformation

The isolation of the Arabidopsis *dgat1-1* (originally referred to as *as11*) mutants has been described previously (Katavic *et al.*, 1995). These mutants were transformed with the construct p2S.*Ea*DAcT (Durrett *et al.*, 2010) using the floral dip method (Clough and Bent, 1998). Transgenic plants were identified by selecting T_1 seeds on MS media containing 30 μg/mL hygromycin. Individual lines homozygous for single genomic insertions were generated through similar selection at the T_2 and T_3 generations.

Camelina transformation

All plasmids used for Camelina transformation were derived from the binary vector pBinGlyRed3 (http://www.camelinagene.org/). This vector contains the visual selectable marker *Discosoma* sp.

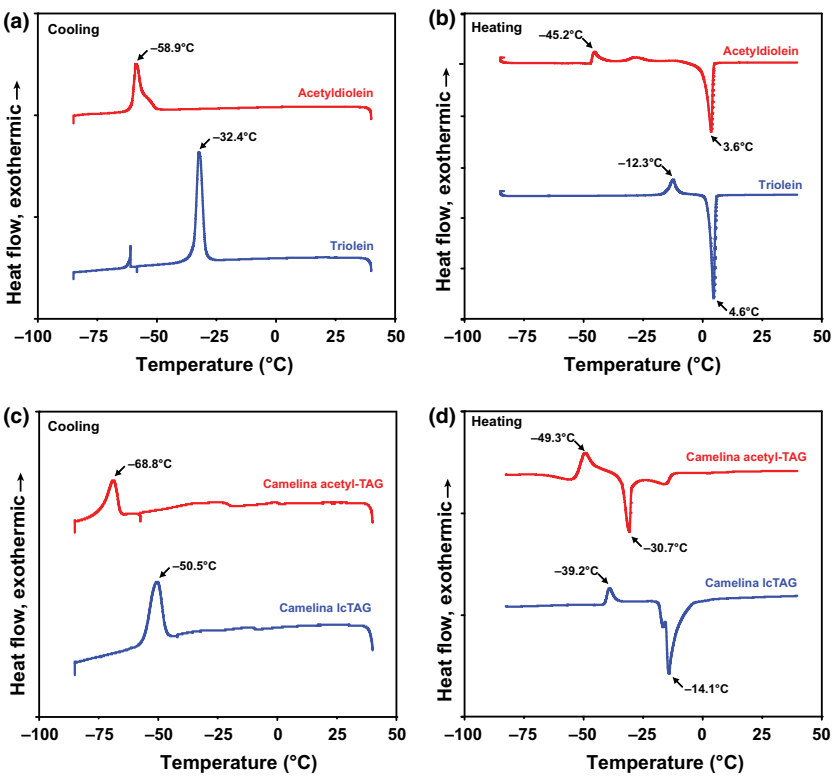

Figure 5 Differential scanning calorimetry (DSC) of acetyl-TAGs and lcTAGs. (a,b) Representative cooling and heating curves for synthetic acetyldiolein and triolein standards. (c,d) Representative cooling and heating curves for acetyl-TAGs purified from transgenic Camelina expressing *Ea*DAcT and lcTAG purified from wild-type Camelina. The cooling curves show *sn*-3 acetylation reduces the transition temperature of the α-polymorphic exotherm substantially; the most stable β′-polymorphic endotherm on melting is little changed by *sn*-3 acetylation for the standards but is lower in Camelina acetyl-TAG versus lcTAG samples. Three scans with different samples were performed for each type of oil with very similar results; these curves show one representative scan. For clarity, the cooling and heating curves are shown separately. The magnitude of the heat flow in each panel has been scaled different to accentuate the important enthalpy changes; hatch marks represent heat flow units of 1 W/g.

fluorescent protein (DsRed). The soybean glycinin promoter was used to drive seed-specific expression of *Ea*DAcT. The sequence for RNAi suppression contained the two arms of a reverse-complement sequence from the Camelina *DGAT1* or *PDAT1* genes interrupted by the *pdk* intron from pHANNIBAL (Wesley *et al.*, 2001). This entire hairpin forming sequence was synthesized by GeneArt and was expressed under the control of the *Brassica napus* oleosin promoter. All T-DNA constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into wild-type *C. sativa* 'Celine' with a floral vacuum infiltration method (Lu and Kang, 2008). T_1 transformant seeds were selected by their dsRED fluorescence. The transgene segregation for fifty T_2 seeds was determined based on the ratio of red and nonred fluorescent seeds. Some lines passed the chi-square test for a 3 : 1 segregation ratio, while others displayed higher ratios suggesting multiple copies of inserts in the genome. To determine acetyl-TAG production in T_2 seeds, null segregants were removed from homozygous and heterozygous seeds by eliminating seeds without dsRED fluorescence.

Camelina field growth

To provide enough seeds for Camelina field trial, seeds from independent transformation events that displayed the highest Acetyl-TAG/lcTAG ratios and no obvious seed morphology phenotypes were amplified in a greenhouse for two sequential generations. Homozygous T_3 plants were selected based on dsRED segregation. T_4 seeds were planted at the Michigan State University Agronomy Farm in four replicates and randomized 8×3 m^2 plots sown in early May and harvested from late July to early August 2013.

Soybean transformation

Native or codon-optimized *Ea*DAcT was placed under control of the seed-specific ß-conglycinin promoter (Allen *et al.*, 1989) with the *Bar* gene used as the selection marker for both constructs. The codon-optimized sequence was synthesized by GeneArt (sequence deposited as GenBank accession KM233194). The T-DNA binary vectors harbouring native or codon-optimized *Ea*DAcT are designated pPTN1074 and pPTN1090, respectively. These vectors were mobilized into *A. tumefaciens* strain EHA101 via tri-parental mating. Agrobacterium-mediated cotyledonary node transformation method was used for soybean transformation as previously described (Zhang *et al.*, 1999). Transformants and progenies were grown under 14-h photoperiod and 28/26 °C day/night temperatures in a greenhouse.

Lipid analysis

Total seed lipids were extracted using a modified hexane–isopropanol extraction method (Li *et al.*, 2006). Tripentadecanoin, and in some cases acetyldiheptadecanoin, was added as internal standards. Total seed lipids were separated on K6 silica TLC plates (Whatman) using a 70:30:1 hexane:diethyl ether:acetic acid (v/ v/ v) solvent system. The relative proportions of acetyl-TAG and lcTAG were estimated by spraying with primuline (0.01% in 80% acetone) and visualized under UV light. Bands corresponding to acetyl-TAG and lcTAG fractions of lines with high acetyl-TAG: lcTAG ratios were recovered by scraping and transmethylated directly using a modified acid-catalysed method (Li *et al.*, 2006). Care was taken to minimize oxidation of polyunsaturated fatty acids by adding butylated hydroxytoluene (BHT) and reducing the time for the high-temperature transmethylation reaction. The fatty acid methyl esters were analysed by gas chromatography using a DB23 capillary column with a flame ionization detector (Li-Beisson *et al.*, 2013). Acetyl-TAG and lcTAG levels were quantified by summing the area of fatty acid methyl ester (FAME) peaks in the chromatogram after correcting for the FID response (Christie, 1991) and normalizing to the added internal standards. Moles of acetyl-TAGs were calculated by dividing moles of FAME by two; for lcTAGs, moles of FAME were divided by three. Total oil content per seed dry weight (adjusted for seed moisture content) was determined gravimetrically in triplicate on oil extracts of 50 seeds. For ESI-MS, neutral lipids were isolated by passage of the total lipid extract through a small silica column with 99:1 (v/ v) chloroform: methanol. Samples were analysed on a Waters (Milford, MA) Quattro micro mass spectrometer as described previously (Bates *et al.*, 2009). Quantification of the signal included corrections for the effects of acyl chain length and number of double bonds on the signal strength (Han and Gross, 2001).

Physical property analyses

Camelina field-grown seeds (400–550 g), either wild type or containing acetyl-TAGs, were extracted three times with two volumes of hexane by grinding with a 35-mm Polytron probe for 15 min. The mixture was vacuum-filtered through filter paper and a 1-cm layer of Celite® 545 filter aid (Sigma-Aldrich, St. Louis, MO). The extracts were combined, reduced in volume with a rotary evaporator and applied to 700–800 g silica gel in a 90-mm diameter glass column which was eluted with a step gradient of increasing diethyl ether in hexanes: from 100:0, 85:15, 80:20, 75:25, to 70:30 (v/ v). Fractions were collected and analysed with TLC developed in hexane:diethyl ether, 60:40. Column fractions containing only lcTAG (from wild-type seed) or acetyl-TAG (from transgenic seed) were combined and solvent removed with a rotary evaporator. The resulting neat oil was used for physical property measurements. Kinematic viscosity was determined using ASTM method D96 by Iowa Central Fuel Testing Laboratory (Fort Dodge, IA). Heats of combustion were measured using a Parr 1341 Oxygen Bomb Calorimeter. Differential scanning calorimetry (DSC) was performed in triplicate on a TA Instruments model Q2000 calorimeter with a thermal cycle from ambient to 40 °C, and then with data collection occurring as the sample was cooled and reheated across the range of −85 to 40 °C at 1 °C/min. For each DSC, peak onset and peak temperatures were determined directly from the scan by use of the system software. Triolein was obtained from Sigma-Aldrich (St. Louis, MO), and *sn*-3-acetyl-1,2-diolein was synthesized by Bridge Organics (Kalamazoo, MI).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 A phylogenetic tree of representative MBOATs with known substrates.

Figure S2 Arabidopsis *dgat1* mutants expressing *Ea*DAcT accumulate enhanced levels of acTAGs.

Figure S3 Constructs used to express *Ea*DAcT and suppress Camelina acyltransferases.

Figure S4 Soybean seeds expressing *Ea*DAcT accumulate acTAGs.